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TITLE: INHIBITION OF PANCREATIC CANCER CELL PROLIFERATION BY LRH-1
INHIBITORS

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14. ABSTRACT The diagnosis of pancreatic cancer is devastating, with mortality rates nearing its incidence rates. To date, there are no effective targeted anti-pancreatic cancer therapeutics. We propose experiments that will explore function of an essential regulator of pancreatic development, the nuclear receptor LRH-1, in pancreatic cancer cells. Our target protein, LRH-1 receptor, is known to control not just one but multiple regulatory mechanisms essential for tumor growth and spread. We propose to find selective and potent compounds that inhibit LRH-1 activity in human pancreatic cancer cells, blunting their growth, proliferation and spread. The identified novel inhibitors of LRH-1 could then be developed into a pharmaceutical that will inhibit growth and proliferation of cancer cells in pancreatic tumors.					
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INHIBITION OF PANCREATIC CANCER CELL PROLIFERATION BY LRH-1 INHIBITORS

PI: Robert J. Fletterick, Ph.D.

Project Period 9/15/12 – 9/14/13

Date of Report: 9/14/13

Progress report: Substantial progress has been made towards **Aim 1** and **Aim 2** of this program (**Task 1** and **Task 2** of the approved SOW); experiments related to **Aim 3** (**Task 3** of SOW) are ongoing.

Aim 1 (Task 1): Discovery of small molecules – antagonists of nuclear receptor LRH-1.

As was proposed in our application, we performed computational filtering of over 5 million compounds and tested top ranked hits in the following biophysical and cell biology assays.

Virtual screening was performed against a constructed model of an inactive state of hLRH-1 LBD, as described in our recently published research article by Benod et al (1). For computational docking experiments, we used a library of 5.2 million commercially available molecules from the ZINC database. Following visual inspection of the resulting top-ranked 500 compounds (~0.01% of the initial library content), twelve molecules were selected for experimental evaluations.

From these verification experiments, we identified two compounds - **Cpd (3)** (1-(3'-{1-[2-(4-morpholinyl)ethyl]-1H-pyrazol-3-yl}-3-biphenyl)ethanone; shown docked in the LRH-1 ligand binding pocket in Fig. 1A) and **Cpd (3d2)** (4-(3-{1-[2-(dimethylamino)ethyl]-1H-pyrazol-3-yl}phenyl)-N,N,5, 6-tetramethyl-2-pyrimidinamine; shown docked in LRH-1 pocket in Fig. 1B) - that bind to LRH-1 LBD directly. The evidence for direct binding was obtained using two independent methods – *Differential Scanning Fluorimetry* (**Task 1a**) and *Surface Plasmon Resonance* (SPR) using quantitative Biacore based assay (**Task 1b**). Analyses of the SPR response isotherms estimated the corresponding **K_d values of 1.5 ± 0.3 μM for Cpd (3)** and **1.8 ± 0.4 μM for Cpd (3d2)** (Fig. 2, for details see (1) and attached .pdf file).

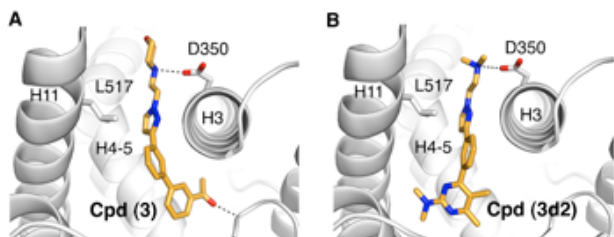


Fig. 1. Predicted mode of binding for Cpd (3) (A) and Cpd(3d2) (B). A cartoon model for LRH-1 polypeptide chain in the vicinity of the ligand-binding pocket is shown in gray, with structural elements forming the pocket indicated. Docked compounds are shown as color-coded stick models. Side chain of Asp350 predicted to facilitate binding interactions of LRH-1 with the ligands is indicated.

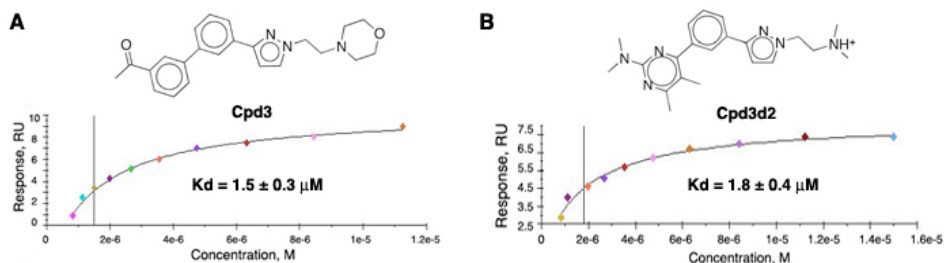


Fig. 2. Evaluation of binding affinity of compounds (3) (A) and (3d2) (B) in the Biacore based direct binding assay. The corresponding chemical structures of compounds are indicated at the top of the panels.

Transcription assay: To verify that these compounds not only bind LRH-1 but also deactivate the receptor upon binding, the transcriptional activity of LRH-1 was assessed in the absence and the presence of Cpd (3) and

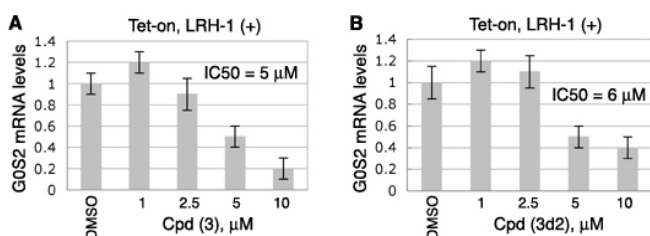


Fig. 3. Effects of Cpd (3) and Cpd (3d2) on transcriptional activity of LRH-1. HEK293 cells expressing LRH-1 receptor (following induction with tetracycline (Tet-on), LRH-1 (+)) were treated with different concentrations of either Cpd (3) (A) or Cpd (3d2) (B). Following 24 h treatments, levels of mRNA for G0S2 (LRH-1 target gene) have been evaluated by qPCR relative to control (cells treated with DMSO).

Cpd (3d2). These experiments showed that treatments with each individual compound lowered the levels of mRNA for G0S2 gene (a transcriptional target of LRH-1) (Fig. 3); the corresponding IC₅₀ values were determined to be 5 ± 1 μM (Fig. 3A) and 6 ± 1 μM (Fig. 3B). To prove that the observed effects by compounds are LRH-1 mediated, the analogous experiments were performed in non-induced HEK293 cells, which do not express the receptor; under these conditions, no significant changes in the levels of G0S2 transcripts were detected in cells treated with either compound compared to the control (1).

Assessing specificity of Cpd (3) and Cpd (3d2): We examined whether the identified LRH-1 inhibitors exert any effects on transcriptional activities of other nuclear receptors. Using previously published methods, transactivation by three different nuclear receptors – steroidogenic factor 1 (SF-1, a close structural and functional analogue of LRH-1) as well as more distant thyroid hormone receptor beta (TRβ) and androgen receptor (AR) - was assessed in the absence and the presence of Cpd (3) and (3d2). These transcriptional studies presented no evidence of any specific, probe-mediated changes in the transcriptional activities of any of the tested receptors (1). Based on these data, we conclude that the identified inhibitors bind to the LRH-1 receptor and inhibit its transcriptional activity preferentially.

Aim 2 (Task 2): Testing LRH-1 inhibitors in pancreatic cancer cells.

Because multiple LRH-1 gene targets (including cyclin E1 (Cyc E1), cyclin D1 (Cyc D1) and C-Myc genes) are known to control cell growth and proliferation, we investigated whether treatments of cells with the identified receptor antagonist affects cell proliferation *in vitro* (**Task 2a**). Our previous work (2) demonstrated that selective inhibition of LRH-1 transcription by siRNA arrests growth and proliferation of human pancreatic cancer cells. This receptor-mediated anti-proliferative effect was observed in four different pancreatic ductal adenocarcinoma (PDAC) cell lines, including AsPC-1, which express high levels of LRH-1 (2). Our current work shows that treatments of AsPC-1 cells with Cpd (3) and (3d2) result in a similar, dose-dependent inhibition of cell proliferation (Fig. 4A, B; concentrations of Cpd (3) and Cpd (3d2) associated with ~50% inhibition of cell proliferation are indicated).

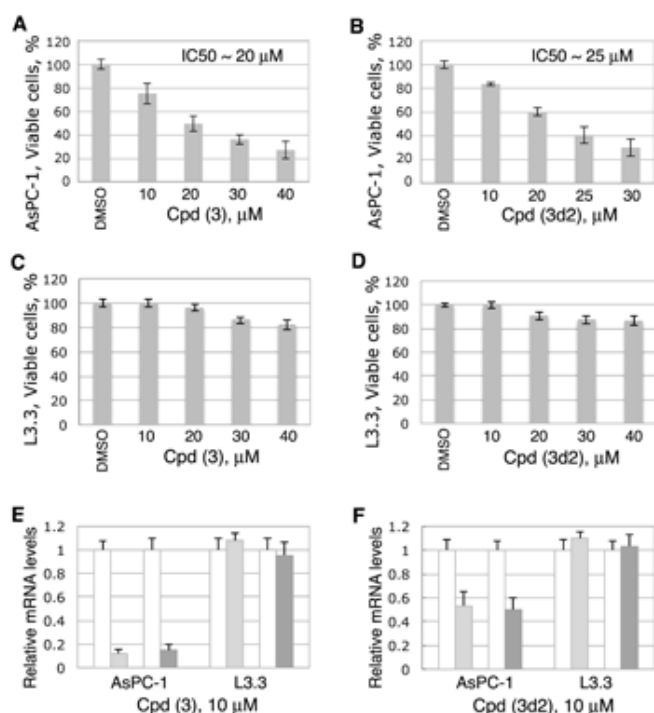


Fig. 4. LRH-1 antagonists inhibit proliferation of pancreatic cancer cells AsPC-1 (LRH-1 positive), but not L3.3 cells (LRH-1 negative). A-D, Cell proliferation rates for both pancreatic cancer cells were measured and compared in the absence and the presence of different concentrations of compounds (3) (A, C) and (3d2) (B, D) relative to control (0.1% DMSO). The corresponding IC₅₀ values are indicated. E, F, Effects of compounds (3) (E) and (3d2) (F) on transcription of the receptor target genes *NR0B2* (encoding SHP) and *CCNE1* (encoding Cyclin E1, Cyc E1) in AsPC-1 and L3.3 cells. Cell samples were analyzed by qPCR for the relative levels of mRNA corresponding to SHP and Cyc E1 following treatments with individual compounds at 10 μM concentration. Controls in white correspond to cells treated with solvent (0.1% DMSO); light and dark gray bars show the levels of mRNA for SHP and Cyc E1 in cells treated with indicated compounds. Data are shown as average of three independent measurements, with experimental errors indicated.

Notably, no significant anti-proliferative effects were observed in pancreatic cancer cells L3.3 (Fig. 4C, D) that do not express LRH-1 receptor at a detectable level (2). In concert with these data, inhibition of transcription of LRH-1 target genes *NR0B2* and *CCNE1* (encoding SHP and Cyclin E1, in light and dark gray, Fig. 4E, F) was detected in AsPC-1 but not in L3.3 cells following these treatments (**Task 2b**). No general cytotoxicity was encountered for either compound at the concentrations used for these experiments (1). These results support the idea that the observed anti-proliferative effects of the probes are receptor-mediated and specific.

Our work demonstrates that human pancreatic ductal adenocarcinoma (PDAC) cells expressing LRH-1 are sensitive to treatments with the receptor specific inhibitors, and that growth and proliferation of LRH-1 positive cancer cells could be markedly decreased following such treatments.

The structure-based identification and characterization of the first LRH-1 specific antagonists is described in our recently published research article by Benod et al (1). This paper reports the use of existing technologies but presents the first specific and potent compounds deriving from computational docking to a non-native protein target structure.

Through ongoing SAR (structure-activity relationship) studies in the PI's lab, we are optimizing the properties of the current LRH-1 antagonists (**Aim 3** of the proposal, **Task 3** of SOW). The goal of this program is to enhance the binding affinities and thus the therapeutic potential of LRH-1 antagonists without compromising their specificity.

Publications derived from this grant:

1. Benod C, Carlsson J, Uthayaruban R, Hwang P, Irwin JJ, Doak AK, Shoichet BK, Sablin EP, and Fletterick RJ (2013) Structure-Based Discovery of Antagonists of Nuclear Receptor LRH-1. *J Biol Chem.* **288**(27):19830-19844. doi: 10.1074/jbc.M112.411686. Epub 2013 May 10.

Other relevant publications:

2. Benod C, Vinogradova MV, Jouravel N, Kim GE, Fletterick RJ, and Sablin EP (2011) Nuclear receptor liver receptor homologue 1 (LRH-1) regulates pancreatic cancer cell growth and proliferation. *Proc Natl Acad Sci USA* **108** (41):16927-16931. doi: 10.1073/pnas.1112047108. Epub 2011 Sep 26.